JOURNAL OF BACTERIOLOGY, Dec. 1988, p. 5848–5854
0021-9193/88/125848-07$02.00/0

Vol. 170, No. 12

Cloning and Sequencing of the Gene Encoding Thermophilic β-Amylase of *Clostridium thermosulfurogenes*

NORIYUKI KITAMOTO,† HIDEO YAMAGATA,* TAKEO KATO,† NORIHIRO TSUKAGOSHI, AND SHIGEZO UDAKA

Department of Food Science and Technology, Faculty of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan

Received 26 May 1988/Accepted 2 September 1988

A gene coding for thermophilic β-amylase of *Clostridium thermosulfurogenes* was cloned into *Bacillus subtilis*, and its nucleotide sequence was determined. The nucleotide sequence suggested that the thermophilic β-amylase is translated from monocistronic mRNA as a secretory precursor with a signal peptide of 32 amino acid residues. The deduced amino acid sequence of the mature β-amylase contained 519 residues with a molecular weight of 57,167. The amino acid sequence of the *C. thermosulfurogenes* β-amylase showed 54, 32, and 32% homology with those of the *Bacillus polymyxa*, soybean, and barley β-amylases, respectively. Twelve well-conserved regions were found among the amino acid sequences of the four β-amylases. To elucidate the mechanism rendering the *C. thermosulfurogenes* β-amylase thermophilic, its amino acid sequence was compared with that of the *B. polymyxa* β-amylase. The *C. thermosulfurogenes* β-amylase contained more Cys residues and fewer hydrophilic amino acid residues than the *B. polymyxa* β-amylase did. Several regions were found in the amino acid sequence of the *C. thermosulfurogenes* β-amylase, where the hydrophobicity was remarkably high as compared with that of the corresponding regions of the *B. polymyxa* β-amylase.

β-Amylase is an exo-type enzyme that hydrolyzes the α-1,4-glucosidic linkages from the nonreducing end of starch and produces maltose with the β-anomeric configuration. Although α-amylases, endo-type enzymes, are widely distributed in various kinds of organisms, β-amylases are known to be produced only by plants and certain bacteria. Extensive studies on α-amylases or α-amylase genes revealed that α-amylases of diverse origins, mammalian to bacterial, have common well-preserved regions including active centers (3, 6, 14), but relatively little is known about the structure-function relationships of β-amylases.

β-Amylases are secreted by several species of the genus *Bacillus*, including *B. polymyxa* (15, 19), *B. cereus* (20, 27, 29), and *B. megaterium* (4), and by *Clostridium thermosulfurogenes* (5). However, only the gene for the *B. polymyxa* β-amylase has been cloned, by us (9) and by Friedberg and Rhodes (2), and the nucleotide sequence of its 5′ portion has been determined (9). As for plant β-amylases, the primary structures of those of barley (10) and soybean (17) have been determined from the respective nucleotide sequences.

The β-amylase of *C. thermosulfurogenes*, a thermophilic and anaerobic bacterium, is unique among the β-amylases so far examined and may be useful in industry, since it is stable and optimally active at 80 and 75°C, respectively (5). To determine the primary structure of the heat-stable β-amylase and to elucidate the mechanism conferring thermophilicity on the enzyme, we cloned the β-amylase gene of *C. thermosulfurogenes* into *Bacillus subtilis* and determined its nucleotide sequence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *C. thermosulfurogenes* ATCC 33743 was grown in TYE medium (5) supplemented with 1% soluble starch at 60°C under anaerobic conditions. *B. subtilis* 1A289 (*amyE sacA321 aroI906 metB5*; *Bacillus* Genetic Stock Center, Ohio State University, Columbus) was grown at 37°C in antibiotic medium no. 3 (M3; Difco Laboratories, Detroit, Mich.). Solid media contained 1.5% agar. Plasmid pHW1B was described previously (32). Erythromycin (10 μg/ml) was added to the medium for the growth of plasmid-carrying strains.

Preparation of DNA and transformation. *C. thermosulfurogenes* DNA was isolated by the method of Saito and Miura (24). Plasmid DNA was isolated from *B. subtilis* by the method of Tanaka et al. (30). Transformation of *B. subtilis* was performed as described by Chang and Cohen (1).

Cloning procedures for the *C. thermosulfurogenes* β-amylase gene. *C. thermosulfurogenes* DNA was partially digested with *Sau*3AI. Fragments of 3 to 9 kilobase pairs (kb) in length were isolated by preparative agarose gel electrophoresis followed by electroelution. Then 0.3 μg of the isolated fragments and 0.1 μg of pHW1B DNA cleaved with *Bam*HI were joined with T4 DNA ligase and used for transformation of *B. subtilis* 1A289 to erythromycin resistance. Transformants were replicated onto M3 agar plates supplemented with 0.3% soluble starch and 10 μg of erythromycin per ml. After growth at 37°C for 24 h, the plates were incubated at 65°C for 3 h; then β-amylase-positive clones were detected by staining with a 10 mM I_2–KI solution.

Preparation of crude enzyme solutions and the β-amylase assay. The culture supernatants of *C. thermosulfurogenes* grown for 72 h and *B. subtilis* 1A289 carrying pNK1, pNK2, or pNK3 grown for 24 h as described above were used as enzyme sources. Ammonium sulfate was added to 80% saturation to the supernatants. The precipitates were collected by centrifugation at 9,000 × *g* for 10 min at 4°C, dissolved in 5 ml of 50 mM acetate buffer (pH 6.0), and then used as crude enzyme solutions after dialysis against the same buffer. β-Amylase activity was assayed by the method of Murao et al. (19). One unit was defined as the activity causing the formation of 1 μmol of maltose from soluble starch in 1 min.

Purification of β-amylase produced by *C. thermosulfuro-*

* Corresponding author.
† Present address: Food Research Institute, Aichi Prefectural Government, 2-1, Shinpukuji-cho, Nishi-ku, Nagoya 451, Japan.

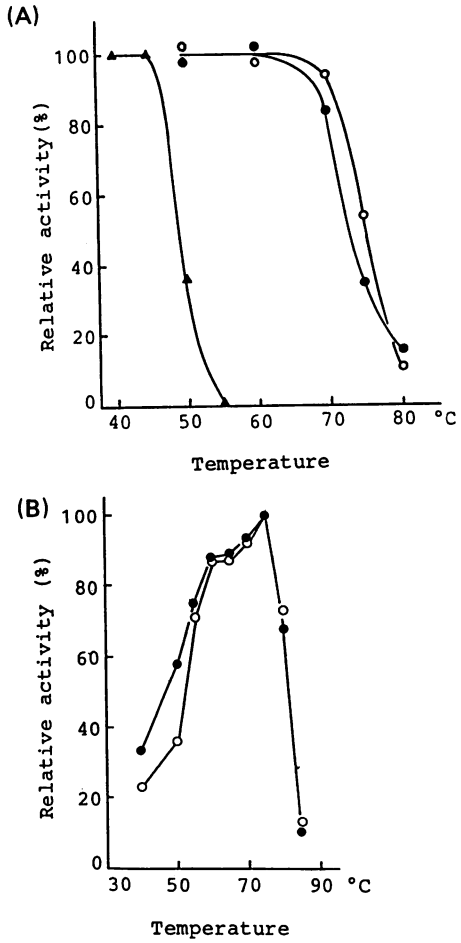

FIG. 1. Heat stability and optimum temperature of the β-amylase produced by *B. subtilis* carrying plasmid pNK1. (A) Heat stability of β-amylases. Crude enzymes prepared from culture supernatants of *B. subtilis* 1A289 carrying pNK1 (●), *C. thermosulfurogenes* (○), and *B. polymyxa* (▲) were incubated at the indicated temperature for 30 min. The residual β-amylase activity was determined at 60°C except the last enzyme, which was assayed at 37°C as described under Materials and Methods. (B) Effect of temperature on the activities of the β-amylases. The activities of the β-amylases prepared from *B. subtilis* 1A289 carrying pNK1 (●) and *C. thermosulfurogenes* (○) were measured for 30 min at each temperature.

***genes* and determination of its NH_2-terminal amino acid sequence.** The ammonium sulfate precipitate prepared from a culture supernatant of *C. thermosulfurogenes* as described above was dissolved in and then dialyzed extensively against 50 mM acetate buffer (pH 6.0) containing 10 mM sodium thioglycolate and 5 mM $CaCl_2$. The dialysate was subjected to chromatography on a DEAE-Sepharose CL6B column (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with the buffer described above. The unadsorbed fraction was concentrated by ultrafiltration with a Diaflo ultrafilter PM10 (Amicon Corp., Arlington Heights, Ill.) and then subjected to gel filtration through Bio-Gel A 1.5m (Bio-Rad Laboratories, Richmond, Calif.). β-Amylase-positive fractions were pooled, lyophilized, and dissolved in the same buffer as above. After dialysis against the same buffer, the sample was subjected to high-performance liquid chromatography on Asahipak GS-520P (Japan Spectroscopic Co.) by using the same buffer for elution. β-Amylase-positive fractions were pooled and mixed with 5 volumes of cold acetone.

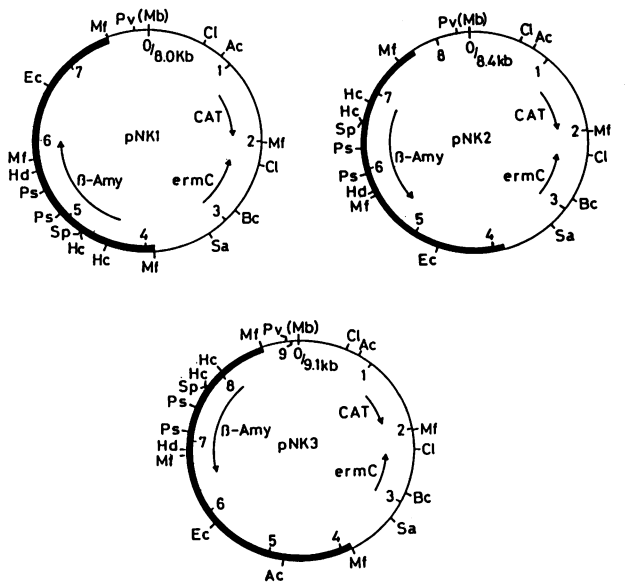

FIG. 2. Restriction maps of pNK1, pNK2, and pNK3 carrying the *C. thermosulfurogenes* β-amylase gene. The thick lines represent *C. thermosulfurogenes* DNA. ermC, CAT, and β-amy with arrows indicate the positions and the direction of transcription of the erythromycin resistance gene, the chloramphenicol acetyltransferase gene, and the β-amylase gene, respectively. Restriction sites: Cl, *Cla*I; Ac, *Acc*I; Mf, *Mfl*I; Bc, *Bcl*I; Sa, *Sal*I; Hc, *Hin*cII; Sp, *Sph*I; Ps, *Pst*I; Hd, *Hin*dIII; Ec, *Eco*RI; Mb, *Mbo*I. For the *Mbo*I site, only that used as a start point for indication of distances is shown.

The precipitate obtained on centrifugation was dissolved in the above-described buffer and dialyzed extensively against water. β-Amylase thus purified gave a single band on sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. The NH_2-terminal amino acid sequence of the purified β-amylase was determined with an ABI477A/120A protein sequencer (Applied Biosystems). The combined amino acid repetitive yield was approximately 90%.

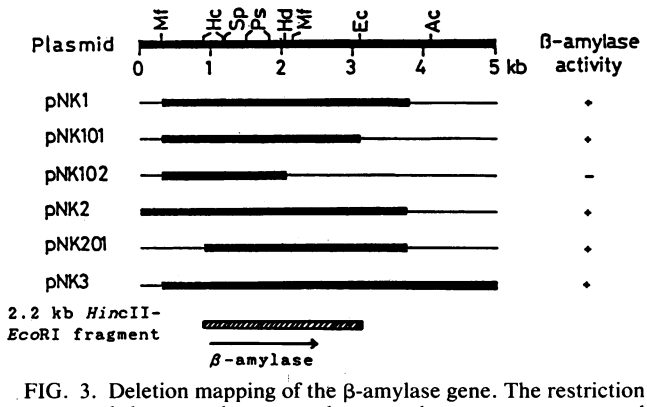

FIG. 3. Deletion mapping of the β-amylase gene. The restriction map around the β-amylase gene shown at the top was constructed by combining the maps of the inserts in pNK1, pNK2, and pNK3. pNK101, pNK102, and pNK201 are deletion derivatives obtained from pNK1, pNK1, and pNK2, respectively. Closed bars represent *C. thermosulfurogenes* DNA carried by these plasmids. The ability (+) or inability (−) of each plasmid to direct extracellular β-amylase synthesis is shown to the right. The bar (▨) at the bottom denotes the *Hin*cII-*Eco*RI fragment containing the β-amylase gene. For abbreviations, see the legend to Fig. 2.

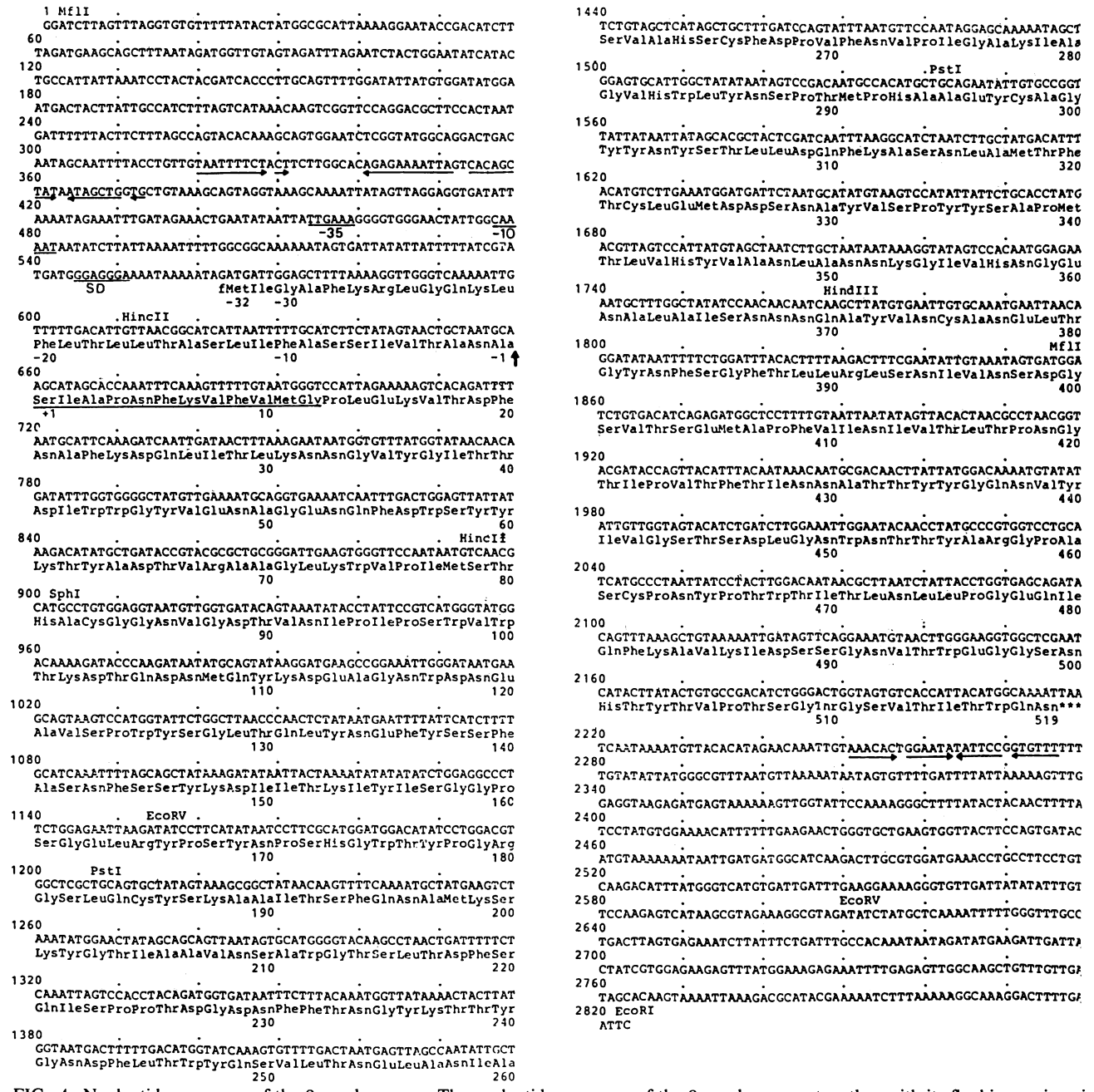

FIG. 4. Nucleotide sequence of the β-amylase gene. The nucleotide sequence of the β-amylase gene together with its flanking regions is shown. Only the sequence of antisense strand is shown. The deduced amino acid sequence of the precursor β-amylase is shown below the nucleotide sequence. The possible −35 and −10 sequences in the promoter region and the possible ribosome-binding site, SD, are underlined. Palindromic sequences are shown by horizontal arrows below the sequence. The amino acid sequence identical with that of the NH_2 terminus of the purified mature *C. thermosulfurogenes* β-amylase is underlined. The possible signal peptide cleavage site is indicated by a vertical arrow. The restriction sites used in this study are shown above the nucleotide sequence.

DNA sequence analysis. The DNA sequence was determined by the dideoxy-chain termination method of Sanger et al. (25). Vector plasmids pUC118 and pUC119 and phage KO7 (31) were used to prepare single-stranded plasmid DNA. Sequencing was performed for the entire lengths of both strands, and all of the ends of the DNA fragments used overlapped with one another.

Other analytical procedures. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (13). Thin-layer chromatography was carried out to identify sugars with propanol-water (70:30) as the solvent system by the multiple ascending method. Silver nitrate was used to detect sugars. Hydropathy of β-amylases was calculated by the method of Kyte and Doolittle (11) with a span of five amino acid residues.

RESULTS AND DISCUSSION

Cloning of the thermophilic β-amylase gene of *C. thermosulfurogenes* into *B. subtilis*. The *Sau*3AI fragments of *C.*

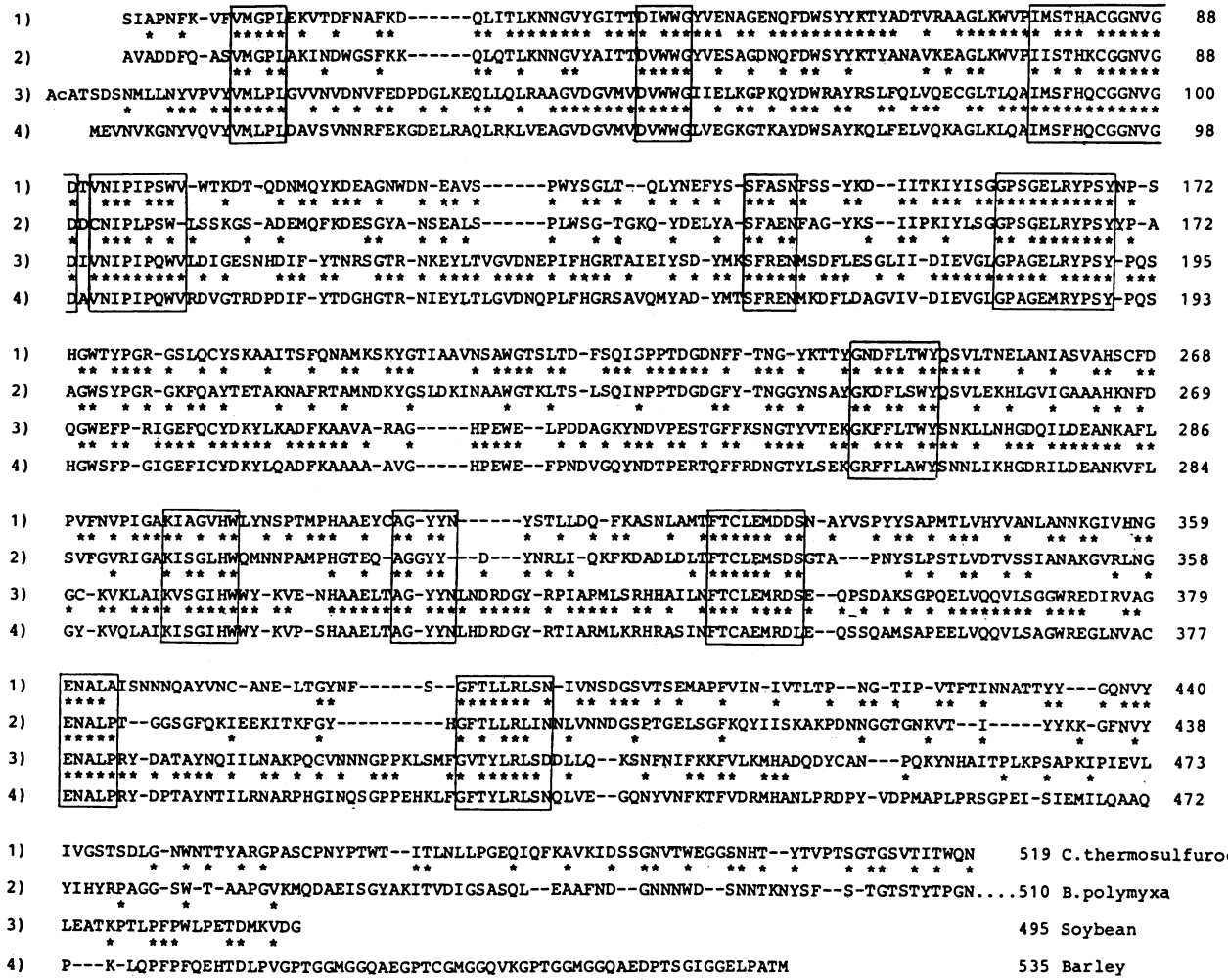

FIG. 5. Comparison of the amino acid sequences of various β-amylases. The sequences of the following mature β-amylases are compared: 1, *C. thermosulfurogenes* (this study); 2, *B. polymyxa* (9); 3, soybean (17); 4, barley (10). For the *B. polymyxa* β-amylase, the amino acid sequence of the NH_2-terminal 510 residues, which is sufficient to constitute an enzymatically active fragment, is used (see text). The sequences are aligned to obtain maximum homology. Dashes in the amino acid sequences denote the deletion of corresponding residues. Identical residues are denoted by asterisks between the sequences. Regions well conserved in all β-amylases are boxed.

thermosulfurogenes DNA of 3 to 9 kb in length were inserted into the *Bam*HI site of plasmid pHW1B (32) and then used to transform *B. subtilis* 1A289, an amylase-deficient strain. The amylase-producing transformants were detected on starch-containing plates by pouring on an KI-I_2 solution, as described in Materials and Methods. Among 6,000 transformants examined, 3 clones (NK1, NK2, and NK3) had reddish transparent zones around their colonies. The crude enzyme solutions prepared from the culture supernatants of these three clones showed starch-degrading activity. The degradation product was exclusively maltose. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by renaturation and activity staining in situ (12), the enzymes from the three clones migrated to the same position as the purified *C. thermosulfurogenes* β-amylase with a apparent molecular weight of about 55,000 (data not shown). Furthermore, the enzymes showed the same heat stability and optimal temperature as those of the enzyme prepared from *C. thermosulfurogenes* (Fig. 1). These results indicate that the amylase-positive clones isolated here contained the β-amylase gene of *C. thermosulfurogenes*.

Restriction analysis of the plasmids carried by the β-amylase-positive clones. The restriction maps of the plasmids harbored by the three amylase-positive clones are shown in Fig. 2. The three plasmids, pNK1, pNK2, and pNK3, contained inserts of 3.6, 4.0, and 4.7 kb in length, respectively. A 3.5-kb *Mfl*I-*Sau*3AI fragment was common among the three plasmids. Deletion analysis of the plasmids showed that a 2.2-kb *Hin*cII-*Eco*RI fragment within the 3.5-kb fragment shown at the bottom of Fig. 3, contained the β-amylase gene. Southern blot analysis (28) of the *C. thermosulfurogenes* genome, in which the cloned DNA fragments were used as hybridization probes, indicated the existence of a single β-amylase gene in the genome and that no deletion or DNA rearrangement in the gene had occurred during the cloning procedures (data not shown).

Nucleotide sequence of the β-amylase gene. The nucleotide sequence of the 2,823-base-pair *Mfl*I-*Eco*RI fragment containing the *Hin*cII-*Eco*RI fragment mentioned above was determined (Fig. 4). We found only one long open reading frame, which started from ATG at nucleotide number 564 and ended in TAA at nucleotide 2,217. The open reading frame encoded a polypeptide of 551 amino acid residues. Since the amino acid sequence deduced from the DNA sequence contained the NH_2-terminal amino acid sequence of the mature β-amylase of *C. thermosulfurogenes* deter-

TABLE 1. Comparison of the amino acid compositions of mature β-amylases

Amino acid	No. (%) of amino acid residues			
	C. thermosulfurogenes		*B. polymyxa*[a]	
	Total	Nonhomologous	Total	Nonhomologous
Charged residues	69 (13.3)	27 (13.3)	99 (19.4)	57 (24.8)
Hydrophilic residues	105 (20.2)	37 (15.5)	132 (25.9)	65 (28.3)
Hydrophobic residues	244 (47.0)	102 (42.7)	241 (47.3)	100 (43.5)
Neutral residues	170 (32.8)	100 (41.8)	137 (26.9)	65 (28.3)
Gly	41 (7.9)	6 (2.5)	58 (11.4)	23 (10.0)
Ala	39 (7.5)	22 (9.2)	44 (8.7)	27 (11.7)
Val	33 (6.4)	19 (7.9)	19 (3.7)	6 (2.6)
Leu	29 (5.6)	12 (5.0)	31 (6.1)	15 (6.3)
Ile	29 (5.6)	16 (6.7)	25 (4.9)	12 (5.0)
Met	9 (1.7)	4 (1.7)	7 (1.5)	2 (0.9)
Phe	22 (4.2)	8 (3.3)	21 (4.1)	7 (3.0)
Trp	16 (3.1)	5 (2.1)	13 (2.6)	2 (0.9)
Pro	26 (5.0)	10 (4.2)	23 (4.5)	6 (2.6)
Ser	46 (8.9)	30 (12.5)	47 (9.2)	29 (12.6)
Thr	51 (9.8)	28 (11.7)	32 (6.3)	9 (3.9)
Asn	51 (9.8)	31 (13.0)	39 (7.7)	19 (8.3)
Gln	15 (2.9)	6 (2.5)	16 (3.1)	7 (3.0)
Cys	7 (1.4)	5 (2.1)	3 (0.6)	1 (0.4)
Asp	22 (4.2)	9 (3.8)	29 (5.7)	16 (7.0)
Glu	15 (2.9)	6 (2.5)	18 (3.5)	9 (3.9)
Lys	19 (3.7)	6 (2.5)	37 (7.3)	24 (10.4)
His	8 (1.5)	4 (1.7)	7 (1.4)	3 (1.3)
Arg	5 (1.0)	2 (0.8)	8 (1.6)	5 (2.2)
Tyr	36 (7.0)	10 (4.2)	33 (6.5)	8 (3.5)

[a] The NH_2-terminal 510 residues of the mature *B. polymyxa* enzyme are used (see the text).

mined chemically (amino acids +1 through +12; underlined in Fig. 4), the open reading frame should represent a structural gene for the β-amylase. The amino acid sequence from amino acids −32 to −1 showed characteristics typical of signal peptides of secretory precursors, i.e., two positively charged residues near the NH_2 terminus followed by a hydrophobic stretch (8).

Sequences homologous to the consensus sequences for the σ^{43} RNA polymerase of *B. subtilis* (TTGACA at position −35 and TATAAT at position −10 [18]) and a possible ribosomal binding site (SD), a sequence homologous to the 3′ end of *B. subtilis* 16S rRNA (16), were found upstream from the ORF (TTGAAA at nucleotides 454 through 459, CAAAAT at nucleotides 477 through 482, and GGAGGGA at nucleotides 545 through 551; underlined in Fig. 4). A palindromic sequence that could form a stable stem-and-loop structure (ΔG = −19.5 kcal [ca. 81.6 kJ]/mol; nucleotides 2,251 through 2,276) followed by an A+T-rich sequence is characteristic of rho-independent transcriptional terminators of *Escherichia coli* (23). From these findings, we consider that the β-amylase of *C. thermosulfurogenes* is translated from a monocistronic mRNA as a secretory precursor with a signal peptide of 32 amino acid residues. We deduce that the mature *C. thermosulfurogenes* β-amylase consists of 519 amino acid residues with a molecular weight of 57,167. The molecular weight agrees well with that estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified enzyme described above.

Comparison of the amino acid sequences of various β-amylases. Figure 5 compares the amino acid sequences of the mature β-amylases of *C. thermosulfurogenes* (this study), *B. polymyxa* (9), soybean (17), and barley (10). Since the β-amylase of *B. polymyxa* is synthesized as a large molecule with a molecular weight higher than 100,000, the amino acid sequence of the NH_2-terminal 510 amino acid residues, which is sufficient to constitute an enzymatically active fragment (9), was used in this study. We found that 54, 32, and 32% of the amino acid residues of the *C. thermosulfurogenes* β-amylase were homologous to those of the *B. polymyxa*, soybean, and barley β-amylases, respectively. The homology was higher on the NH_2-terminal side than on the COOH-terminal side. Twelve regions relatively well conserved among the four β-amylases are boxed in Fig. 5. These regions include the three highly conserved sequences of β-amylases found by Mikami et al. (17) and might be important for the expression of enzyme activity. Since β-amylases so far examined are all susceptible to sulfhydryl reagents, Cys-88 and Cys-322 of the *C. thermosulfurogenes* β-amylase, located within the highly conserved regions mentioned above, should play important roles in the catalytic reaction or in maintenance of the enzyme structure.

Based on the homology between the amino acid sequences of the *C. thermosulfurogenes* and *B. polymyxa* β-amylases, the structures of the two enzymes may be basically similar. The unique thermophilicity of the former enzyme should be due to its nonhomologous amino acid residues. Table 1 compares the amino acid compositions of the two enzymes. The *C. thermosulfurogenes* β-amylase contains seven Cys residues, whereas the *B. polymyxa* β-amylase only contains three. The increased number of Cys residues might be responsible for the heat stability of the former enzyme by generating an additional disulfide bond(s) as shown in the case of stabilization of T4 lysozyme (22) and subtilisin BPN′ (21) toward thermal inactivation. The number of hydrophilic amino acid residues is smaller in the *C. thermosulfurogenes* β-amylase (105 residues, 20% of the total residues) than in the *B. polymyxa* β-amylase (132 residues, 26% of the total residues). The difference is more evident when the nonhomologous residues of the two enzymes are compared. Only 15% of the nonhomologous residues are hydrophilic in the

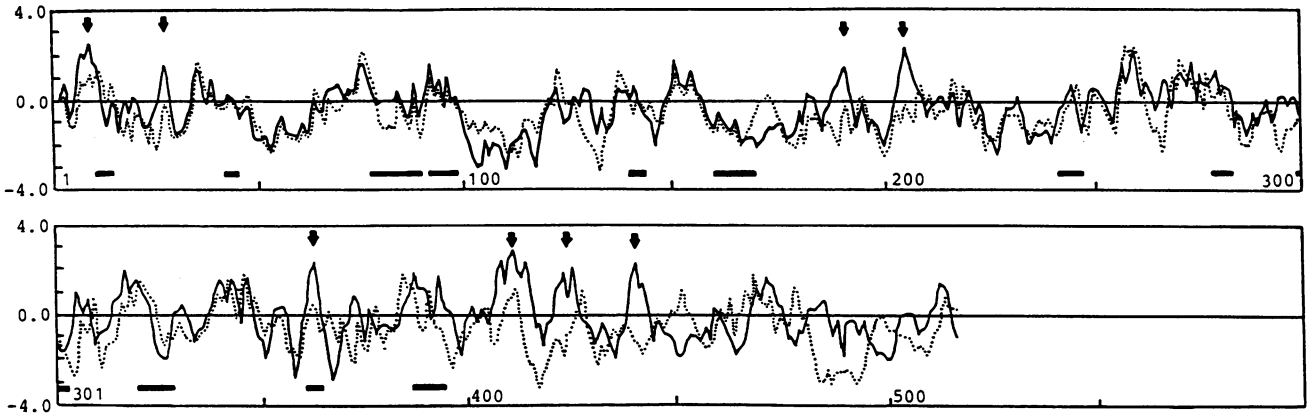

FIG. 6. Comparison of the hydropathy profiles of the *C. thermosulfurogenes* and *B. polymyxa* β-amylases. The profile of the mature β-amylase of *C. thermosulfurogenes* (——) and that of the NH_2-terminal 510 amino acid residues of the *B. polymyxa* β-amylase (· · · ·) are compared. The abscissa of each panel shows the amino acid number. The ordinate shows the average hydrophobicity (positive ordinate) or hydrophilicity (negative ordinate) of 5 amino acid residues. Arrows indicate regions where the hydrophobicity of the *C. thermosulfurogenes* enzyme is markedly higher than that of the *B. polymyxa* enzyme. Horizontal bars at the bottom of each panel denote well-conserved regions found in the amino acid sequences of four β-amylases of different origins (Fig. 5).

former, whereas 28% of those of the latter are hydrophilic (Table 1). The lower content of hydrophilic amino acid residues might increase the internal hydrophobicity of the enzyme molecule so that it folds into a heat-stable form with stronger internal packing in an aqueous solution, as is the case in the alteration of the thermostability of the *Bacillus stearothermophilus* neutral protease (7). Figure 6 compares the hydropathy profiles of the *C. thermosulfurogenes* and *B. polymyxa* β-amylases. The hydrophobicity of the former was higher than that of the latter as a whole and especially in the several regions indicated by arrows. These regions might represent those internally packed in the β-amylase molecule as mentioned above. We are at present examining such possibilities by means of site-directed mutagenesis of the cloned β-amylase gene and by construction of chimeric genes between the two enzymes and analysis of the heat stability of the gene products. The primary structure of the thermophilic β-amylase presented here will also be useful for investigation of the phylogenetic relationships among various amylases of different origins (26).

ACKNOWLEDGMENTS

We are grateful to Bunzo Mikami, Yuhei Morita, and Chikafusa Fukazawa for informing us of the primary structure of the soybean β-amylase before publication.

This investigation was supported in part by a fund from Japan Foundation for Applied Enzymology.

LITERATURE CITED

1. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168**:111–115.
2. **Friedberg, F., and C. Rhodes.** 1986. Cloning and characterization of the beta-amylase gene from *Bacillus polymyxa*. J. Bacteriol. **165**:819–824.
3. **Friedberg, F.** 1983. On the primary structure of amylases. FEBS Lett. **152**:139–140.
4. **Higashihara, M., and S. Okada.** 1974. Studies on β-amylase of *Bacillus megaterium* strain no. 32. Agric. Biol. Chem. **38**:1023–1029.
5. **Hyun, H. H., and J. G. Zeikus.** 1985. General biochemical characterization of thermostable extracellular β-amylase from *Clostridium thermosulfurogenes*. Appl. Environ. Microbiol. **49**: 1162–1167.
6. **Ihara, H., T. Sasaki, A. Tsuboi, H. Yamagata, N. Tsukagoshi, and S. Udaka.** 1985. Complete nucleotide sequence of a thermophilic α-amylase gene: homology between prokaryotic and eukaryotic α-amylases at the active sites. J. Biochem. **98**:95–103.
7. **Imanaka, T., M. Shibazaki, and M. Takagi.** 1986. A new way of enhancing the thermostability of proteases. Nature (London) **324**:695–697.
8. **Inouye, M., and S. Halegoua.** 1980. Secretion and membrane localization of proteins in *Escherichia coli*. Crit. Rev. Biochem. **7**:339–371.
9. **Kawazu, T., Y. Nakanishi, N. Uozumi, T. Sasaki, H. Yamagata, N. Tsukagoshi, and S. Udaka.** 1987. Cloning and nucleotide sequence of the gene coding for enzymatically active fragments of the *Bacillus polymyxa* β-amylase. J. Bacteriol. **169**:1564–1570.
10. **Kreis, M., M. Williamson, B. Buxton, J. Pywell, J. Hejgaard, and I. Svendsen.** 1987. Primary structure and differential expression of β-amylase in normal and mutant barleys. Eur. J. Biochem. **169**:517–525.
11. **Kyte, J., and R. F. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
12. **Lacks, S. A., and S. S. Springhorn.** 1980. Renaturation of enzymes after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. J. Biol. Chem. **255**:7467–7473.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
14. **Long, C. M., M.-J. Virolle, S.-Y. Chang, S. Chang, and M. J. Bibb.** 1987. α-Amylase gene of *Streptomyces limosus*: nucleotide sequence, expression motifs, and amino acid sequence homology to mammalian and invertebrate α-amylases. J. Bacteriol. **169**:5745–5754.
15. **Marshall, J. J.** 1974. Characterization of *Bacillus polymyxa* amylase as an exo-acting (1→4)-α-D-glucan maltohydrolase. FEBS. Lett. **46**:1–4.
16. **McLaughlin, J. R., C. L. Murray, and J. C. Rabinowitz.** 1981. Unique features in the ribosome binding site sequence of the gram-positive *Staphylococcus aureus* β-lactamase gene. J. Biol. Chem. **256**:11283–11291.
17. **Mikami, B., Y. Morita, and C. Fukazawa.** 1988. Primary structure and function of β-amylase. Seikagaku (Tokyo) **60**:211–216. (In Japanese.)
18. **Moran, C. P., N. Lang, S. F. LeGrice, G. Lee, M. Stephens, A. L. Sonenshein, J. Pero, and R. Losick.** 1982. Nucleotide sequences that signal the initiation of transcription and transla-

tion in *Bacillus subtilis*. Mol. Gen. Genet. **186**:339–346.
19. **Murao, S., K. Ohyama, and M. Arai.** 1979. β-Amylases from *Bacillus polymyxa* no. 72. Agric. Biol. Chem. **43**:719–726.
20. **Nanmori, T., R. Shinke, K. Aoki, and H. Nishira.** 1983. Purification and characterization of β-amylase from *Bacillus cereus* BQ 10-S1 SpoII. Agric. Biol. Chem. **47**:941–947.
21. **Pantoliano, M. W., R. C. Ladner, P. N. Bryan, M. L. Rollence, J. F. Wood, and T. L. Poulos.** 1987. Protein engineering of subtilisin BPN′: enhanced stabilization through the introduction of two cysteines to form a disulfide bond. Biochemistry **26**: 2077–2082.
22. **Perry, L. J., and R. Wetzel.** 1984. Disulfide bond engineered into T4 lysozyme: stabilization of the protein toward thermal inactivation. Science **226**:555–557.
23. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Genet. **13**:319–353.
24. **Saito, H., and K. Miura.** 1963. Preparation of transforming DNA by phenol treatment. Biochim. Biophys. Acta **72**:619–629.
25. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
26. **Satoh, H., H. Nishida, and K. Isono.** 1988. Evidence for movement of the α-amylase gene into two phylogenetically distant *Bacillus stearothermophilus* strains. J. Bacteriol. **170**:1034–1040.
27. **Shinke, R., H. Nishira, and N. Mugibayashi.** 1974. Isolation of β-amylase producing microorganisms. Agric. Biol. Chem. **38**: 665–666.
28. **Southern, E.** 1979. Gel electrophoresis of restriction fragments. Methods Enzymol. **68**:152–176.
29. **Takasaki, Y.** 1976. Production and utilization of β-amylase and pullulanase from *Bacillus cereus* var. *mycoides*. Agric. Biol. Chem. **40**:1515–1522.
30. **Tanaka, T., M. Kuroda, and K. Sakaguchi.** 1977. Isolation and characterization of four plasmids from *Bacillus subtilis*. J. Bacteriol. **129**:1487–1494.
31. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153**:3–11.
32. **Yamagata, H., T. Adachi, A. Tsuboi, M. Takao, T. Sasaki, N. Tsukagoshi, and S. Udaka.** 1987. Cloning and characterization of the 5′ region of the cell wall protein gene operon in *Bacillus brevis* 47. J. Bacteriol. **169**:1239–1245.